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Article

The Broad Antibacterial Activity of the Natural Antibody Repertoire Is Due to Polyreactive Antibodies

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SUMMARY

Polyreactive antibodies bind to a variety of structurally unrelated antigens. The function of these antibodies, however, has remained an enigma, and because of their low binding affinity their biological relevance has been questioned. Using a panel of monoclonal polyreactive antibodies, we showed that these antibodies can bind to both Gram-negative and Gram-positive bacteria and acting through the classical complement pathway can inhibit bacterial growth by lysis, generate anaphylatoxin C5a, enhance phagocytosis, and neutralize the functional activity of endotoxin. Polyreactive antibody-enriched, but not polyreactive antibody-reduced, IgM prepared from normal human serum displays antibacterial activity similar to that of monoclonal polyreactive IgM. We conclude that polyreactive antibodies are a major contributor to the broad antibacterial activity of the natural antibody repertoire.

INTRODUCTION

What are now known as natural antibodies were first described nearly 100 years ago (Tauber and Podolsky, 1997). Innumerable studies since have shown that serum containing these antibodies has bactericidal activity, and in fact, to distinguish antigen-induced antibodies from natural antibodies, serum is routinely diluted 10- to 50-fold before testing to reduce the background activity of the natural antibodies (Notkins, 2004). Natural antibodies, however, have remained an enigma to immunologists because they are found in serum in the apparent absence of antigenic stimulation and are present in newborns and germ-free animals. Absorption of serum with a specific protein or bacterium can result in the loss of antibody activity not only to the antigen used for absorption, but also to other unrelated proteins or bacteria (Gordon and Carter, 1932; Michael et al., 1962). Adding further to the enigma of natural antibodies is the observation that many natural antibodies react with a variety of normal host proteins, suggesting that some of these antibodies are autoantibodies, which would seem to contradict the well-accepted view that the host is immunologically tolerant of most self antigens. The fact that normal serum contains millions of different Ig molecules, all in small quantities, has made it difficult to characterize these antibodies.

With the advent of hybridoma technology in the mid-1970s, it became possible to prepare large quantities of individual Ig molecules. Analysis of these monoclonal Ig molecules, prepared from normal individuals, revealed that many were polyreactive; that is, they could bind to a variety of structurally unrelated self (e.g., proteins, lipids, carbohydrates, DNA) and nonself (e.g., bacteria, viruses) antigens (Haspel et al., 1983; Dighiero et al., 1983; Casali and Notkins, 1989; Prabhakar et al., 1984; Burastero et al., 1988). These polyreactive antibodies bind to antigens with low affinity (Kd = 10^{-3} to 10^{-7} mol l^{-1}) as compared to monoreactive antibodies (Kd = 10^{-7} to 10^{-11} mol l^{-1}), and each polyreactive antibody has a distinct antigen-binding pattern that can vary for different antigens by as much as 1000-fold (Haspel et al., 1983; Dighiero et al., 1983; Casali and Notkins, 1989; Prabhakar et al., 1984; Burastero et al., 1988). Many of the polyreactive antibodies have a germline or near-germline sequence and are primarily IgM, but some are also IgG and IgA. Contrary to the classic ''lock and key'' rigid-structure hypothesis of antigen-antibody interaction, the antigen-binding pocket of polyreactive antibodies, perhaps because of their germline configuration, are believed to be more flexible and therefore can accommodate different antigenic configurations (Notkins, 2004). The B cells that make these antibodies can be identified by the binding of fluorescein-labeled antigens to the polyreactive B cell receptors on their surface (Zhou and Notkins, 2004; Wang et al., 2001). Some of those polyreactive antigen-binding B (PAB) cells have a B-1-positive, whereas others have a B-1-negative, phenotype (Zhou and Notkins, 2004). In the newborn up to 50% of peripheral blood B lymphocytes can make polyreactive antibodies. In the adult the number is reduced to between 15% and 20% (Chen et al., 1998).

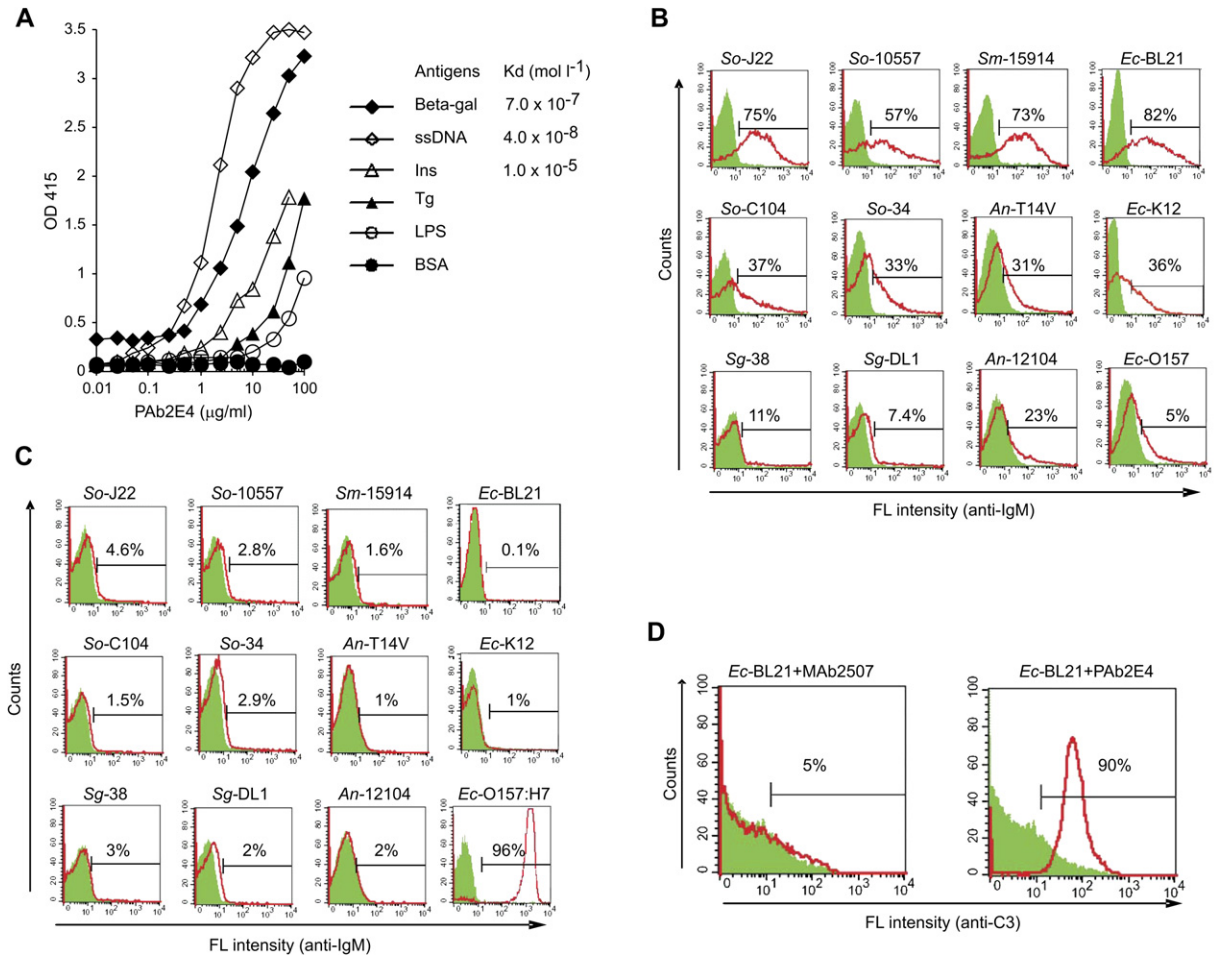

Figure 1. Properties of Polyreactive Antibody 2E4

(A) Dose-dependent binding of 2E4 to different antigens as measured by ELISA and the calculated dissociation constants (Kd).

(B) Polyreactive antibody 2E4 (red line) binds to a variety of Gram-positive and Gram-negative bacteria.

(C) Nonpolyreactive MAb2507 (red line) only binds to its cognate antigen, *E. coli* O157: H7.

(D) Polyreactive antibody 2E4 (right panel), but not nonpolyreactive MAb2507 (left panel), fixes complement as measured by FACS analysis with anti-C3.

So, *S. oralis*; *Sm*, *S. mitis*; *Sg*, *S. gordonii*; *An*, *A. naeslundii*; *Ec*, *E. coli.*

Polyreactive antibodies therefore are a major component of the natural antibody repertoire.

Natural antibodies have long been thought to be a first line of defense against bacterial and viral infections (Casali and Notkins, 1989), but the biological function of polyreactive antibodies has never been determined. In fact, because of their low affinity their biological relevance has been questioned. The present experiments were initiated to determine whether polyreactive antibodies have antibacterial activity.

RESULTS

Properties of Polyreactive Antibody 2E4

Mouse hybridomas were prepared, cloned, and screened for polyreactivity. Polyreactive antibody 2E4 was chosen for detailed study. Of the antigens screened the strongest binding was to ssDNA and β-gal with weaker binding to insulin, Tg, and LPS (Figure 1A). The dissociation constants (Kd) for ssDNA, β-gal, and insulin were 4.0×10^{-8}, 7.0×10^{-7}, and 1.0×10^{-5}, respectively. As with other polyreactive antibodies (Haspel et al., 1983), 2E4 bound to a variety of organs and cell types as determined by indirect immunofluorescence (data not shown). Gene sequencing revealed that 2E4 is an IgM antibody with a germline sequence that is 99.7% identical to J558.8 (Table S1 in the Supplemental Data available with this article online).

Polyreactive Antibody 2E4 Binds to a Variety of Different Bacteria

The binding of 2E4 to a variety of Gram-positive and Gram-negative bacteria, including different serotypes of three antigenically distinct genera, was determined by FACS analysis. Representative data (Figure 1B) show that 2E4 binds strongly to some bacteria (*Streptococcus*

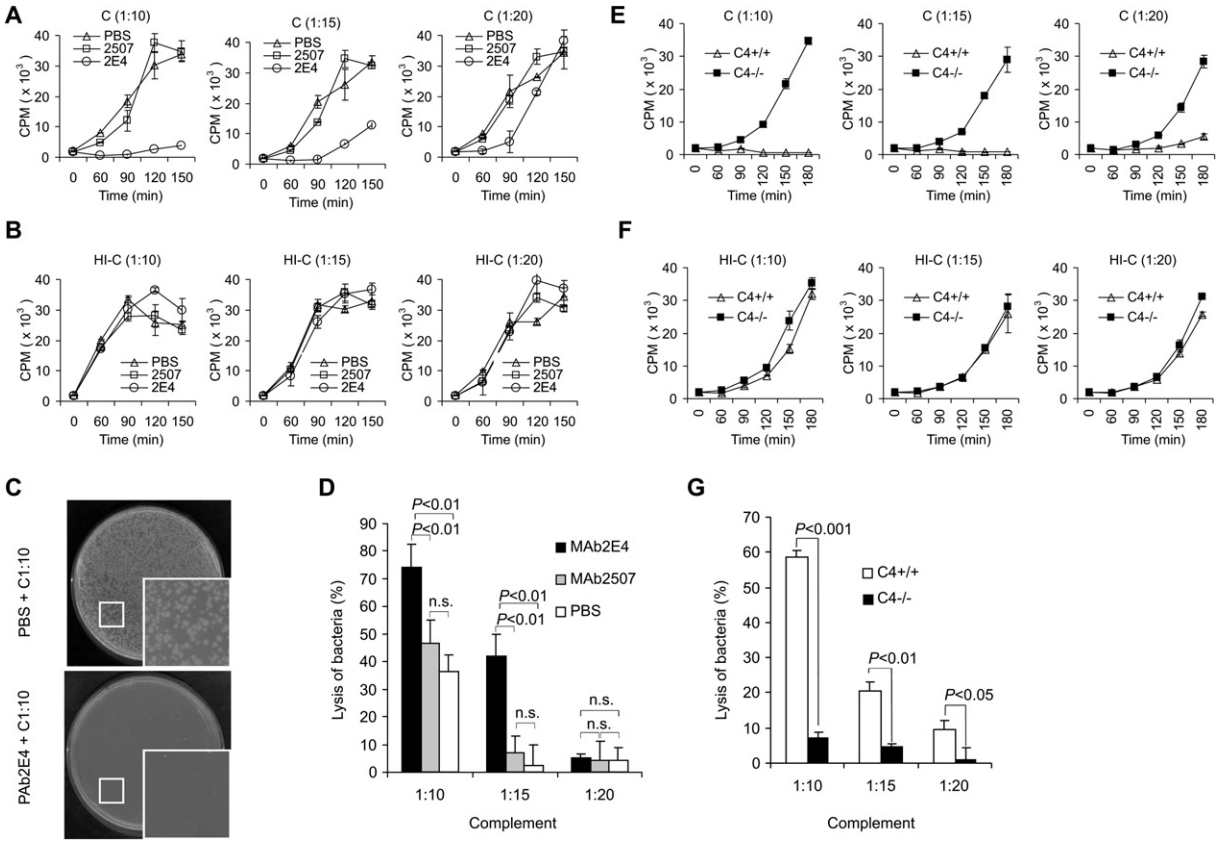

Figure 2. Polyreactive 2E4 Is Bactericidal in the Presence of Complement

(A and B) Growth of 2E4-treated *E. coli* BL21 as measured by the incorporation of ^{3}H-TdR in the presence of different concentrations of untreated (A) and heat-inactivated (B) complement.

(C) Bacteria colony formation on agar plates in the presence of complement with or without 2E4.

(D) Lysis of 2E4-treated bacteria as measured by the release of ^{3}H-TdR in the presence of different concentrations of complement.

(E and F) Growth of 2E4-treated bacteria as determined by the incorporation of ^{3}H-TdR in the presence of different concentrations of $C4^{+/+}$ or $C4^{-/-}$ serum (E) or heat-inactivated $C4^{+/+}$ or $C4^{-/-}$ serum (F).

(G) Lysis of 2E4-treated *E. coli* BL21 as measured by the release of ^{3}H-TdR in the presence of different concentrations of $C4^{+/+}$ or $C4^{-/-}$ serum. Bars represent SD.

oralis J22, *Streptococcus oralis* 10557, *Streptococcus mitis* 15914, and *E. coli* BL21), moderately to other bacteria (*Streptococcus oralis* C104, *Streptococcus oralis* 34, *Actinomyces naeslundii* T14V, and *E. coli* K12), and weakly or not at all to still other bacteria (*Streptococcus gordonii* 38, *Streptococcus gordonii* DL1, *Actinomyces naeslundii* 12104, and *E. coli* O157: H7). In contrast, monoclonal antibody MAb2507 reacted only to its cognate antigen *E. coli* O157: H7 (Figure 1C).

Polyreactive Antibody 2E4 Fixes Complement and Inhibits *E. coli* Growth

Bacteria (*E. coli* BL21) that had been incubated with polyreactive antibody 2E4 or the nonbinding monoreactive antibody MAb2507 were incubated with complement. The fixation of complement was determined by FACS analysis with an antibody to the third component of complement (C3). As seen in Figure 1D, complement bound strongly to the polyreactive antibody-treated bacteria, but not to the nonbinding monoreactive antibody-treated bacteria.

The effect of complement on the growth of bacteria treated with polyreactive antibody 2E4 was determined by measuring the incorporation of ^{3}H-thymidine. At a 1:10 dilution of complement, there was little if any incorporation of ^{3}H-thymidine into bacteria as compared to bacteria that had been treated with PBS or with MAb2507 (Figure 2A). At higher dilutions of complement the growth-inhibitory effect decreased, and with heat-inactivated complement (Figure 2B) there was no inhibition of growth.

The inhibitory effect of 2E4 and complement also was demonstrated by plating the 2E4-treated and untreated bacteria on agar plates and evaluating the number and size of the bacterial colonies. Bacteria treated with 2E4 and complement showed little if any colony formation as compared to bacteria treated with PBS and complement (Figure 2C).

To determine if the inhibition of growth was due to bacterial lysis, the release of ^{3}H-TdR from radiolabeled *E. coli* BL21 was measured. Figure 2D shows that considerably

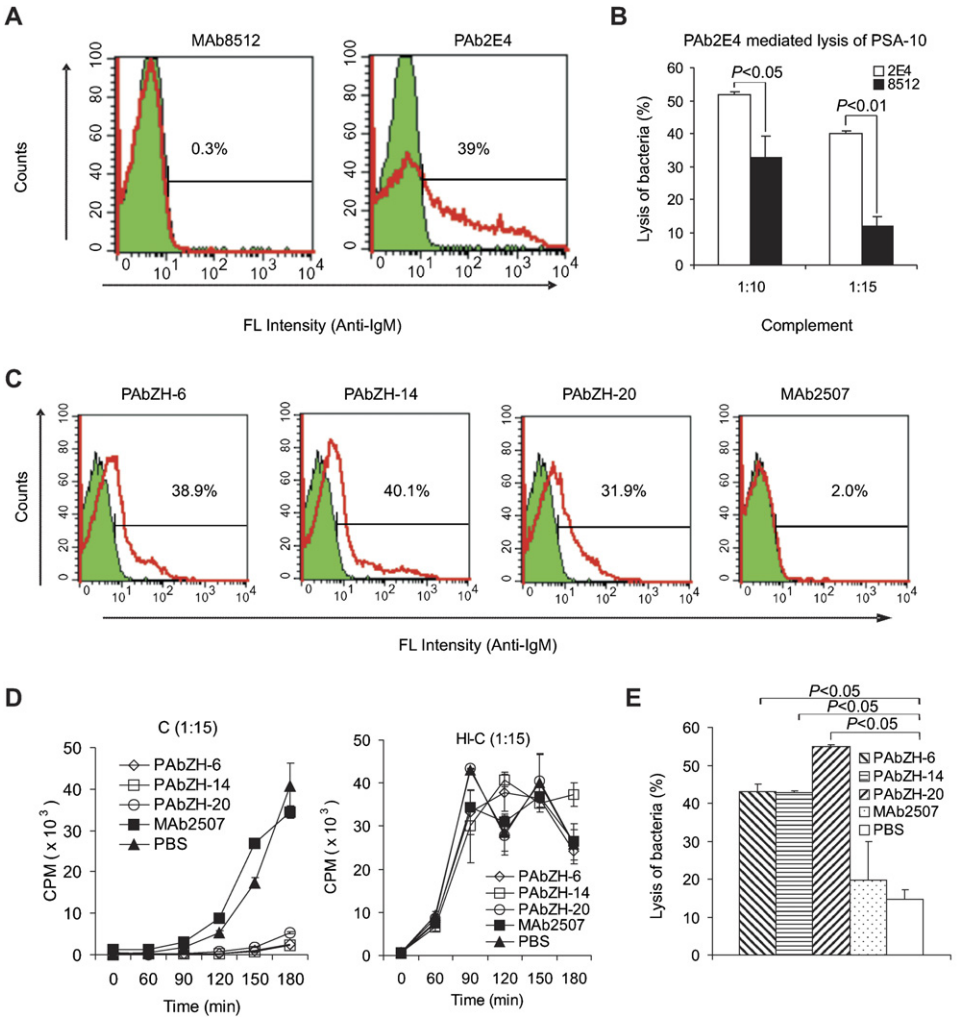

Figure 3. Lysis of *Pseudomonas aeruginosa* by Polyreactive Antibody 2E4 and *E. coli* BL21 by Polyreactive Antibodies ZH-6, ZH-14, and ZH-20 in the Presence of Complement

(A) PAb2E4, but not MAb8512, binds to *Pseudomonas aeruginosa* (PSA-10).

(B) Enhanced lysis of 2E4-treated PSA-10 as compared to MAb8512-treated PSA-10 in the presence of two different concentrations of complement as measured by the release of ^{3}H-adenine.

(C) Polyreactive antibodies ZH-6, ZH-14 and ZH-20, but not nonpolyreactive MAb2507, bind to *E. coli* BL21.

(D) Inhibition of growth of antibody-treated *E. coli* BL21 as measured by incorporation of ^{3}H-TdR in the presence of complement (left) or heat-inactivated complement (right).

(E) Lysis of antibody-treated *E. coli* BL21 as measured by the release of ^{3}H-TdR in the presence of complement.

Error bars represent SD.

more ^{3}H-TdR was released from the cells treated with 2E4 than those treated with MAb2507 or PBS and that the effect was complement dependent.

Bactericidal Activity of 2E4 Is Mediated through the Classical Complement Pathway

The classical complement pathway, in contrast to the alternative complement pathway, is antigen-antibody specific and requires C4 (Carroll, 2004). To see if polyreactive 2E4 exerted its growth-inhibitory effect through the classical complement pathway, $C4^{-/-}$ serum was compared to $C4^{+/+}$ serum as a source of complement. Figure 2E shows that the growth of 2E4-treated bacteria, as measured by the uptake of ^{3}H-TdR, was markedly inhibited by $C4^{+/+}$ serum, but not by $C4^{-/-}$ serum or by heat-inactivated $C4^{+/+}$ serum (Figure 2F). Release of ^{3}H-TdR from radiolabeled 2E4-treated bacteria by $C4^{+/+}$ serum, but not by $C4^{-/-}$ serum, showed that the inhibition of growth was due to bacterial lysis mediated through the classical complement pathway (Figure 2G).

Polyreactive Antibody 2E4 Binds to and Lyses *Pseudomonas aeruginosa*

To see if polyreactive antibody 2E4 would inhibit the growth of other bacteria, *Pseudomonas aeruginosa* serotype 10 (PSA-10), was chosen for study. Figure 3A shows

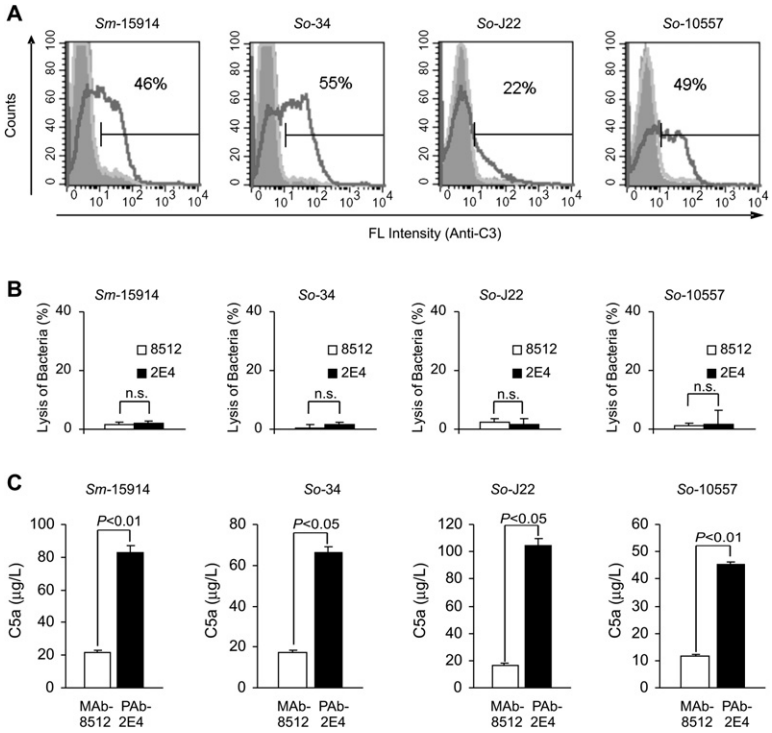

Figure 4. Generation of C5a

Polyreactive antibodies in the presence of complement do not lyse Gram-positive bacteria (i.e., *Streptococcus mitis*, *Streptococcus oralis*) but generate the anaphylatoxin C5a.

(A) Polyreactive antibody 2E4 (black line) fixes complement as measured by FACS analysis with anti-C3 antibody as compared to nonbinding MAb8512 (gray shadow).

(B) Neither polyreactive 2E4-treated nor nonpolyreactive MAb8512-treated Gram-positive bacteria are lysed in the presence of complement (1:10 dilution) as determined by ^{3}H-TdR release.

(C) Monoclonal antibody-treated Gram-positive bacteria were incubated with guinea pig complement (1:10 dilution) followed by incubation with human C5. PAb2E4-treated, but not MAb8512-treated, Gram-positive bacteria generated anaphylatoxin C5a as measured by ELISA.

Error bars represent SD.

that 2E4 binds to PSA-10 as demonstrated by FACS analysis and fixes complement (data not shown). In the presence of complement, polyreactive antibody 2E4, but not nonpolyreactive MAb8512, produced significant lysis of PSA-10 (Figure 3B).

Other Monoclonal Polyreactive Antibodies with Antibacterial Activity

Three additional monoclonal polyreactive antibodies were prepared and tested (Table S1). Monoclonal antibody ZH-6 and ZH-20 were IgMs, belonged to the J558 family, and were 100% and 99.3% identical to J558.2 and J558.13, respectively. Their light chains belonged to the VK-21 family and the VK-12 family, respectively. In contrast, ZH-14 was an IgG3, belonged to the VGAM3.8 (VH9) family, and was 96.8% identical to VH9.1. The light chain belonged to the VK-21 family. These antibodies then were tested for their binding to a variety of antigens and bacteria. In contrast to 2E4 (Figure 1B), all three antibodies showed an intermediate level of binding to *E. coli* BL21 (Figure 3C), but in the presence of complement the three antibodies inhibited the growth of *E. coli* (Figure 3D), which was mediated through bacterial lysis (Figure 3E).

Generation of Anaphylatoxin C5a in the Absence of Bacterial Lysis

Polyreactive antibody 2E4 can bind to a variety of Gram-positive bacteria (Figure 1B) and also fix complement (Figure 4A). However, in contrast to Gram-negative bacteria, Gram-positive bacteria are not lysed (Figure 4B), nor is their growth inhibited (data not shown). Despite the failure to inhibit growth, the interaction of polyreactive antibodies with Gram-positive bacteria, in the presence of complement, generates solid phase C5 convertase and, in turn, the anaphylatoxin C5a, an important chemotaxis factor (Figure 4C).

Polyreactive MAb2E4 Enhances Phagocytosis

The fixation of complement to antigen-antibody complexes can enhance phagocytosis. Bacteria *E. coli* BL21 were labeled with FITC, treated with polyreactive 2E4 or nonbinding MAb2507, and then incubated with complement. Phagocytosis of the bacteria by mouse macrophages was evaluated by confocal microscopy and FACS analysis. As seen in Figure 5A, bacteria treated with polyreactive 2E4 and complement were readily phagocytosed as compared to bacteria treated with nonbinding MAb2507 and complement or with 2E4 alone or complement alone (Figure 5B). Maximum fluorescence (phagocytosis) was detected at 150 min.

Polyreactive Antibodies Neutralize the Functional Activity of Endotoxin

Exposure of human monocytes to endotoxin (LPS) resulted in the production of matrix metalloproteinase 1 (MMP-1) (Zhang et al., 1997). Incubation of LPS from *Salmonella abortus-equi* (SAE) with polyreactive antibody MAb2E4, but not irrelevant MAb8512, reduced the production of MMP-1 (Figure 5C). Similar results were obtained with three other polyreactive antibodies (i.e., ZH-14, ZH-6, and ZH-20) using LPS from three different bacteria (i.e., SAE, *E. coli* O157: H7 and PSA) (Figures

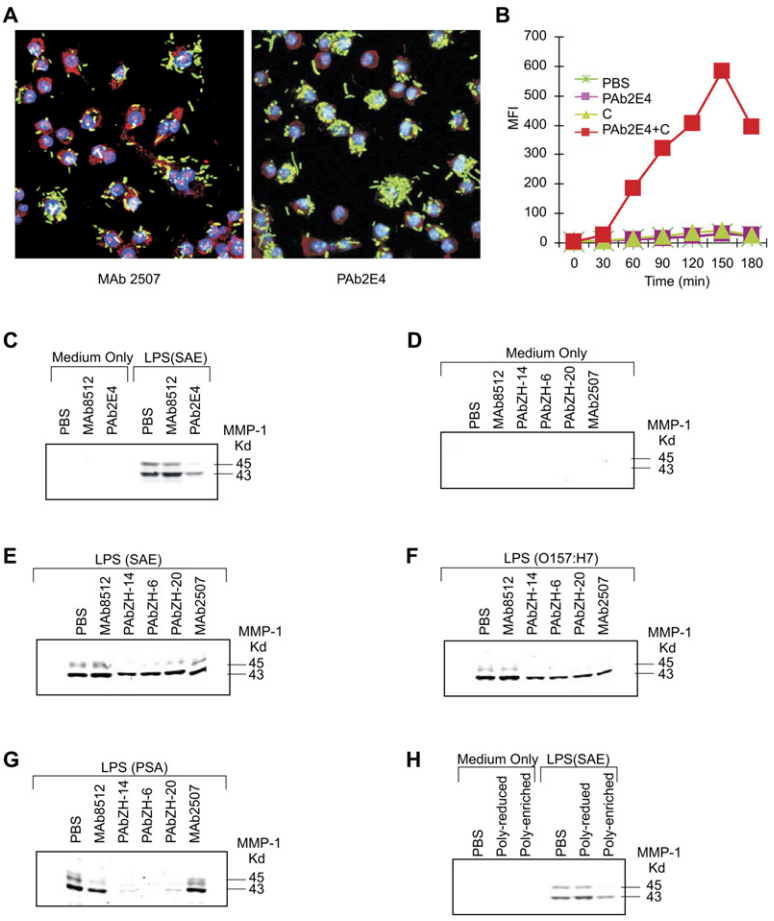

Figure 5. Polyreactive Antibodies Enhance Phagocytosis and Inhibit the Activity of LPS

(A) Immunofluorescence staining shows bacteria *E. coli* BL21 (green) being taken up by macrophages (red) with blue nuclei. Left panel: bacteria treated with MAb2507 and complement. Right panel: bacteria treated with PAb2E4 and complement.

(B) Phagocytosis of 2E4-treated bacteria in the presence of complement, as determined by FACS analysis and measured by mean fluoresce intensity (MFI).

(C) Human monocyte MMP-1 is increased by incubation with LPS (*Salmonella abortus-equi* [SAE]) in the presence of PBS or nonbinding MAb8512 but is decreased by polyreactive 2E4.

(D) MMP-1 is not produced by monocytes incubated with PBS or with different polyreactive (i.e., ZH-14, ZH-6, and ZH-20) or nonpolyreactive MAbs (i.e., 8512 and 2507) in the absence of LPS.

(E–G) Monocyte MMP-1 production is increased by incubation with LPS from SAE (E), *E. coli* O157: H7 (F), or *Pseudomonas aeruginosa* (PSA) (G) in the presence of PBS or nonbinding MAb8512 but is decreased by polyreactive MAbs ZH-14, ZH-6, and ZH-20. Nonpolyreactive MAb2507 decreased only the production of MMP-1 induced by LPS from *E. coli* O157: H7, its cognate antigen (F).

(H) Polyreactive-enriched, but not polyreactive-reduced, human IgM inhibits LPS-induced production of MMP-1.

5E–5G). MAb2507 reduced only the production of MMP-1 by LPS from *Ec*-O157: H7, its cognate antigen (Figure 5F), but not the production of MMP-1 by LPS from SAE or PSA (Figures 5E and 5G). None of the monoclonal antibodies bound directly to monocytes as evaluated by immunostaining and FACS analysis (data not shown). These findings show that polyreactive antibodies can neutralize the functional activity of LPS in the absence of complement.

Polyreactive Antibodies from Serum Are Bactericidal

Polyreactive antibodies from normal serum were enriched by serial passage of affinity-purified human IgM through three different antigen-affinity columns (i.e., ssDNA, β-gal, thyroglobulin). The polyreactive-enriched, as compared to the polyreactive-reduced, IgM bound to a variety of different antigens (Figure 6A), including bacteria (Figure 6B), and in the presence of complement lysed radiolabeled *E. coli* BL21 (Figure 6C). In the absence of complement, polyreactive-enriched, but not polyreactive-reduced, human IgM decreased the production of LPS-induced MMP-1 (Figure 5H). These findings show that polyreactive antibodies from human sera have antibacterial properties similar to monoclonal polyreactive antibodies.

DISCUSSION

Natural antibodies, by definition, are produced in the apparent absence of antigenic stimulation (Tauber and Podolsky, 1997). The most likely explanation for their occurrence is that they are the result of the millions of normally occurring VDJ rearrangements and exist in germline or near-germline configuration. Because of their diversity, the percentage of antibodies in normal serum that are polyreactive has been difficult to determine. However, since up to one-half of the B cells in the cord blood of newborns and one-fifth of the B cells in the peripheral circulation of adult are capable of making polyreactive antibodies, it is evident that these antibodies are highly represented (Chen et al., 1998; Wardemann et al., 2003).

The high representation of polyreactive antibodies raises questions about the nature and function of the nonpolyreactive antibodies in the natural antibody repertoire. Assuming that they too exist in germline or near-germline configuration, it is unclear whether they have sufficient specificity and affinity to be biologically relevant in natural defense against invading pathogens without further antigenic stimulation and somatic mutation. If, in fact, the genes encoding these nonpolyreactive antibodies are not in germline configuration and instead show many somatic mutations, they rightfully should not be considered

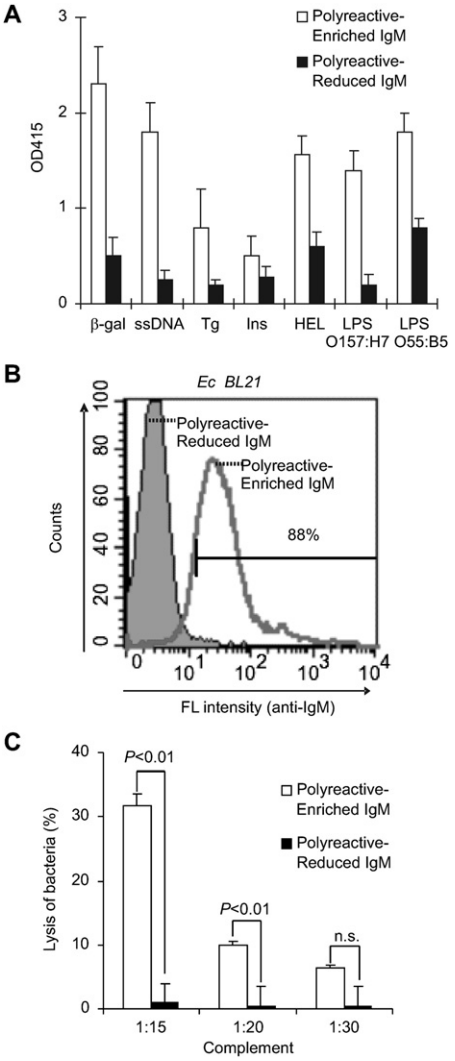

Figure 6. Bactericidal Activity of Polyreactive Antibody-Enriched Human IgM

(A and B) Binding of polyreactive antibody-enriched, as compared to polyreactive antibody-reduced, IgM to different antigens (A) and to *E. coli* BL21 (B).

(C) Polyreactive-enriched, but not polyreactive-reduced, IgM lyses *E. coli* BL21 in the presence of complement as measured by the release of ^{3}H-TdR. Equal concentrations (50 μg/ml) of polyreactive-enriched and polyreactive-reduced IgM were used in all experiments. Error bars represent SD.

part of the natural antibody repertoire. Perhaps more important, even if some of these germline-encoded nonpolyreactive antibodies do have a potential biological function, their concentration in serum as an individual molecular species may be too low to have immediate biological value when confronted with invading pathogens.

In contrast to nonpolyreactive antibodies, the great number and diversity of polyreactive antibodies, with the ability of many of them to recognize the same bacteria and multiple antigens on those bacteria, make them a major component of the natural antibody repertoire and potentially an important first line of defense. This is not meant to negate the possibility that there may be a population of germline-encoded nonpolyreactive natural antibodies directed against evolutionally conserved epitopes found on pathogens such as phosphocholine (Briles et al., 1981; Claflin and Berry, 1988). In fact, such antibodies have been found by hybridoma technology, but in many cases it was not clear whether these antibodies were monoreactive, oligoreactive, or polyreactive (Notkins, 2004); truly encoded by germline sequences; or presented in sufficient concentration in the serum to have biological value.

Polyreactive antibodies are an ancient part of the immune system and have been found in jawed vertebrates going back as far as the shark (Marchalonis et al., 2001; Marchalonis et al., 2002). The ability of polyreactive antibodies to bind to a variety of different antigens suggests that these antibodies represent the humoral component of the innate immune system. In fact, upon entering the host, pathogens will be exposed almost immediately to polyreactive antibodies, since these antibodies are constantly present in serum. This exposure, in the presence of complement, could result in lysis of bacteria, which in turn could lead to the release of ligands (e.g., CpG-DNA) that bind to Toll-like receptors. In this way the innate immune system could be activated (Krieg, 2002). Of particular interest is the fact that, even though the interaction of polyreactive antibodies with Gram-positive bacteria does not result in lysis, it does activate the complement cascade. Cleavage products such as C5a then bind to a whole range of cell surface receptors on myeloid and lymphoid cells and thereby contribute to the host's inflammatory response (Guo and Ward, 2005; Huber-Lang et al., 2006). Thus the initial encounter of polyreactive antibodies with pathogens could serve as a first line of defense and give the adaptive immune system time to be activated.

A variety of studies involving the passive transfer of normal serum to animals that subsequently were challenged with bacteria or viruses showed that normal serum enhanced protection (Ochsenbein et al., 1999; Baumgarth et al., 2000; Quan et al., 1997; Kroese et al., 1996; Boes et al., 1998). To what extent this protection was due to the polyreactive antibodies in the transferred serum, however, has never been determined. The demonstration here that both monoclonal polyreactive antibodies and enriched, but not reduced, polyreactive antibodies prepared from normal serum can lyse bacteria, generate C5a, enhance phagocytosis, and neutralize endotoxin argues that polyreactive antibodies contribute in an important way to the antibacterial activity of normal serum. The effectiveness of polyreactive antibodies depends on the nature of the antigens on the particular pathogen and the titer and binding capacity of the circulating polyreactive antibodies.

In addition to their role in defense, a variety of other functions have been attributed to natural antibodies, including clearance of degraded proteins (e.g., red blood cells, bacteria), the enhancement of immunogenicity by trapping of antigens in lymphoid organs, and

immunoregulatory functions (Ochsenbein et al., 1999; Sapir and Shoenfeld, 2005; Miller and Rodriguez, 1995; Trebst and Stangel, 2006; Robey et al., 2002). In none of these cases, however, has the role of polyreactive antibodies been evaluated. It is of particular interest that the intravenous administration of natural Ig (IVIG) is being used to treat a variety of disorders (Sapir and Shoenfeld, 2005; Siragam et al., 2006). There is no consensus as to how natural Ig works, but again the role of polyreactive antibodies needs to be evaluated.

Although polyreactive antibodies can bind to normal mammalian tissues, under ordinary circumstances, they do not appear to be deleterious to the host, presumably because a variety of regulatory proteins (e.g., factor H, DAF, CR-1) inhibit the lytic action of complement on eukaryotic cells (Zipfel, 2001; Kinoshita et al., 1985; Wagner et al., 2006). Moreover, it is well established that B cells capable of making high-affinity self-reactive antibodies are eliminated from the host early in life by negative selection, but because of their low affinity, there may be little need for the host to eliminate the B cells that make polyreactive antibodies. In fact, because of their low affinity and germline configuration, polyreactive antibodies do not appear to be true autoantibodies and certainly do not fit into the same category as antigen-specific, somatically mutated, high-affinity pathogenic autoantibodies (Brard et al., 1999; Roark et al., 2002; Rahman, 2004; Wellmann et al., 2005). It is possible, however, that the B cells that make polyreactive antibodies might be the precursor population from which at least some high-affinity autoantibodies are derived (Ichiyoshi et al., 1995). There also is some evidence that the polyreactive B cell population may be the result of positive selection. Transgenic mouse models provide support for the positive-selection hypothesis (Julien et al., 2002; Hayakawa et al., 2003; Wong et al., 2002; Hardy and Hayakawa, 2005; Gaudin et al., 2004; Casola et al., 2004), but which self antigens are involved in positive selection of polyreactive B cells is still not clear.

In conclusion, the demonstration that passive transfer of normal sera containing natural antibodies can enhance resistance to bacteria and viruses (Ochsenbein et al., 1999; Boes et al., 1998; Reid et al., 1997) and the demonstration here of the antibacteria, antitoxin (i.e., LPS), and phagocytosis-enhancing effects of polyreactive antibodies raise the intriguing, although still distant, possibility that passive administration of a cocktail of monoclonal polyreactive antibodies might prove useful in the prevention and treatment of some infections. If this turns out to be the case, developing methods to activate in vivo the B cell subset that makes polyreactive antibodies might be of value in broadly enhancing natural resistance during times of bacterial and viral epidemics.

EXPERIMENTAL PROCEDURES

Mice, Cell Lines, and Bacterial Strains

Three- to twelve-week-old BALB/c mice were bred at the NIH animal facility (Bethesda, MD). All experiments were carried out in compliance with institutional guidelines and approved by the NIDCR ACUC (Bethesda, MD). Gram-negative *E. coli* bacteria were cultured in Luria-Bertani (LB) broth, *Pseudomonas aeruginosa* Migula serotypic 10 (PSA-10) were cultured in Nutrient broth (BD234000), and different Gram-positive bacteria (i.e., *S. gordonii*, *S. oralis*, *S. mitis*, and *A. naeslundii*) were cultured in Todd Hewitt broth (Krackeler Scientific, Inc., Albany, NY) until an OD_{600} of 0.3 was reached. Mouse macrophage cell line Raw264.7 was cultured in RPMI1640 with 10% FBS and replaced with serum-free medium before use.

Preparations of Hybridomas

Splenocytes were isolated from 3- to 12-week-old BALB/c mice and fused with Sp2/0-Ag14 myeloma cells (Hartman et al., 1989). Positive clones were subcloned by limiting dilution at least two times. Large amounts of antibody were prepared by culturing hybridoma cells in serum-free CD Hybridoma Medium (Invitrogen, Carlsbad CA). Supernates were concentrated and quantitated by Coomassie Plus Protein Assay Kit (Pierce Biotechnology, Inc, Rockford, IL). In the current study, four new monoclonal polyreactive antibodies (2E4, ZH-6, ZH-14, and ZH-20) were prepared and characterized (Table S1). Two monoclonal monoreactive mouse IgM antibodies (2507 and 8512) were obtained from ATCC (ATCC no. CRL-2507, HB-8512) and used as controls. 2507 is specific for *E. coli* O157: H7 O-antigen, and 8512 is against bacterial cell wall peptidoglycan.

RT-PCR and Sequencing of Variable Regions of Immunoglobulins

Total RNA from hybridomas was isolated with TRIZOL (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol, and the constant and variable regions of IgM and IgG were amplified by PCR using Mouse Ig-Primer Sets from Novagen (EMD Biosciences, Inc., La Jolla, CA). PCR fragments were cloned in pCR2.1 vector (Invitrogen) and sequenced.

ELISA and Affinity Assays

Approximately 10 μg/ml of antigens (β-galactosidase, thyroglobulin, insulin, ssDNA, lipopolysacride, etc.) (Sigma, St. Louis, MO) in coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$) were adsorbed onto Maxisorp microplates (Nalge Nunc, Copenhagen, Denmark). Nonspecific binding sites were blocked by incubation with 1% BSA in phosphate-buffered saline (PBS). Different concentrations of purified monoclonal antibody were loaded in wells. The second antibody (biotin-labeled rat anti-mouse IgM or biotin-labeled polyclonal goat anti-mouse Igs at 2 μg/ml) (BD Pharmigen, San Diego, CA) then was added. The reaction mixture was developed with Avidin-HRP (1:3000 dilution) and ABTS substrate (Zymed Laboratories, South San Francisco, CA), and absorbance was read at 415 nm by an Elisa microplate reader (Molecular Devices Corporation, Sunnyvale, CA). Affinity assays were performed as described (Nakamura et al., 1988) by competitive ELISA, and the dissociation constants (Kd) were calculated according to Friguet et al. (1985).

Affinity Isolation of Human Polyreactive IgM

Affinity columns of ssDNA, β-gal, and thyroglobulin were prepared with HiTrap NHS-activated HP columns (GE Healthcare, Piscataway, NJ) according to the manufacturer's instruction. Purified human IgM (>96% purification) isolated from normal human serum was obtained from Research Diagnostics, Inc., Flanders, NJ. Human IgM was sequentially passed through and eluted from DNA, β-gal, and thyroglobulin columns. The pass-through fractions were designated "polyreactive IgM-reduced" and the eluted fractions "polyreactive IgM-enriched." IgM was eluted by washing with 2 M NaCl and desalted with HiTrap Desalting columns (GE Healthcare). The polyreactive-enriched and polyreactive-reduced IgM fractions were concentrated by Macrosep centrifugation (Pall Life Sciences, Ann Arbor, MI), and the protein concentration was determined by Coomassie Plus Protein Assay Kit (Pierce Biotechnology, Inc., Rockford, IL).

Immunofluorescence Staining and Flow Cytometry Assays

To measure the binding of antibody, 2×10^7 bacteria or 3.0 to 4.0 × 10^5 mammalian cells were preincubated with 1% BSA and 2 mM EDTA at 4°C for 30 min to minimize the effect of nonspecific binding sites. Mammalian cells also were preincubated with 20 μg/ml Mouse Fc Block (BD PharMingen). The cells then were incubated with mouse monoclonal polyreactive or monoreactive antibodies (50 μg/ml) followed by FITC-labeled goat anti-mouse IgM (Sigma-Aldrich Inc, St. Louis, MO) and fixed with 4% paraformaldehyde. Unless indicated otherwise, to measure the binding of complement, antibody-treated bacteria were incubated with 1:15 dilution of guinea pig complement serum (Cedarlane Laboratories Limited, Ontario, Canada) that had been preadsorbed by four rounds of incubation with the bacteria to be tested to eliminate bacteria-binding immunoglobulin that might be present in the guinea pig serum. This was followed by incubation with FITC-labeled goat anti-guinea pig C3 polyclonal antibody (VitroStat Inc., Portland, ME) and fixation with 4% paraformaldehyde. Fluorescence intensity was analyzed by FACSCalibur using CELL Quest software (BD, San Jose, CA).

Bacterial Growth

Bacteria (2×10^7) were treated with 50 μg/ml polyreactive or nonpolyreactive antibodies or PBS, followed by incubation with different dilutions of bacterial adsorbed guinea pig complement serum (to eliminate bacteria-binding Ig) at 37°C for 1 hr. Incorporation of [^{3}H]TdR or [^{3}H]adenine (20 μCi/ml) (GE Healthcare) was used to measure bacterial growth. At different time points, 100 μl of medium containing bacteria was removed, placed in triplicate on 0.45 μm filters of a 96-well filtration plate (Millipore, Bedford, MA), and washed eight times, and the amount of radioactivity remaining on the filters was determined (Microbeta 1450 Trilux liquid scintillation counter, Wallac, Gaithersburg, MD). Growth also was determined by seeding 1:1000 dilution of bacterial cultures onto LB-agar plates, and colonies were counted after overnight incubation at 37°C. All experiments were repeated at least three times.

Bactericidal Assay

E. coli were radiolabeled by culturing in LB broth with 20 μCi/ml [6-^{3}H]Thymidine (GE Healthcare), and PSA-10 were radiolabeled by culturing in nutrient broth (BD234000) with 20 μCi/ml [8-^{3}H]adenine (GE Healthcare). Radio-labeled bacteria (3×10^7), suspended in PBS-BSA, then were incubated with polyreactive or nonpolyreactive monoclonal antibodies (50 μg/ml) to which was added different dilutions of bacterial adsorbed guinea pig complement or bacterial adsorbed $C4^{-/-}$ guinea pig serum (to eliminate bacteria-binding Ig). After incubation at 37°C for 1 hr, the reaction mixtures were transferred to 96-well filtration plates (Millipore) and washed, and the cpm remaining on the filters was determined with a Microbeta 1450 Trilux liquid scintillation counter. Percent lysis was calculated as [(cpm of bacteria treated with control sera) − (cpm of bacteria treated with polyreactive antibody)/(cpm of bacteria treated with control sera)] × 100.

Measurement of C5a

Gram-positive bacteria (2×10^7) were incubated with polyreactive or nonpolyreactive monoclonal antibodies, followed by incubation with 1:10 dilution of guinea pig complement as described above. The bacteria then were washed twice and incubated with 10 μg/ml of complement C5 from human serum (Sigma) at 37°C for 1 hr. Generation of C5a was determined by human C5a ELISA kit (BD Bioscience, San Jose CA).

Opsonization of Bacteria

Bacteria were labeled with FITC using a FluoroTag FITC conjugation kit purchased from Sigma (St. Louis, MO) as described by Weingart et al. (1999). The FITC-labeled bacteria then were incubated with monoclonal polyreactive or nonpolyreactive antibodies or PBS for 30 min, washed, and incubated with 1:15 dilution of bacterial adsorbed guinea pig complement for 30 min. The bacteria then were added to Raw264.7 macrophages cultured in 24-well plates with serum-free medium. At different time points, the cells were trypsinized and fixed with 4% paraformaldehyde, and mean fluorescence intensity determined. Raw264.7 macrophages also were cultured on Lab-Tek Chambered Coverglass (Nunc, Inc. Naperville, IL). The nuclei of Raw264.7 macrophages were prestained (blue) by culturing in 10 μg/ml of Hoechst 33342 (Bis-Benzimide, Sigma) for 90 min, and FITC-labeled bacteria were added. At different time points, 50 μg/ml ethidium bromide was added for 5 min to stain the cytoplasm of the macrophages red. Opsonization was determined by laser scanning confocal microscopy with a Leica TCS-SP2 confocal scanning head mounted on a Leica DMRE-7 (SDK) upright microscope (NA = 1.40; Leica Microsystems Inc., Bannockburn, IL).

Purification of Human Monocytes and Detection of MMP-1 by Western Blot

Human peripheral blood cells were obtained by leukapheresis from normal volunteers at the Department of Transfusion Medicine at the National Institutes of Health. Monocytes were prepared as described previously (Zhang et al., 1997), and the final preparation contained greater than 90% monocytes as determined by morphology, nonspecific esterase staining, and flow cytometry. The purification procedure did not activate the monocytes as demonstrated by the fact that less than 4% of the cells were IL-2R positive, a sensitive marker of monocyte activation. Purified monocytes (5×10^6/ml) then were stimulated with 100 ng/ml LPS or LPS premixed with 3.0 μg/ml antibodies (30 min at 4°C) for 36–48 hr as previously described (Zhang et al., 1997). The supernatant fluids together with BSA (40 μg/ml) were added to cold ethanol and put at −70°C for 15 min. Proteins then were pelleted by microcentrifugation and lyophilized, and then the resuspended proteins were electrophoresed on a 10% Tris-glycine gradient polyacrylamide gel. The proteins then were transferred onto 0.45 μm nitrocellulose, and the blots were incubated overnight with rabbit polyclonal antibodies against matrix metalloproteinase (MMP-1). Western blots were analyzed by the addition of Alexa Fluor 680 goat anti-rabbit IgG (Molecular Probes Inc., Eugene), and the fluorescence was detected with the Odyssey infrared imaging system (LI-COR, Lincoln, NE).

Statistical Analysis

Data are presented as mean ± SD, and all paired comparisons were subjected to a two-tailed Student's t test. Significance was set at a p value of less than 0.05.

Supplemental Data

The Supplemental Data include one supplemental table and can be found with this article online at http://www.cellhostandmicrobe/cgi/content/full/1/1/51/DC1/.

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